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Taxol is a chemotherapeutic compound that shows high response rate in metastatic breast carcinoma. However, some tumors are intrinsically resistant while others develop resistance to taxol in the course of treatment. We hypothesized that resistance may arise from deficiencies in cellular functions that normally potentiate cytotoxicity. To identify the genes involved in taxol response we are using the genetic suppressor element (GSE) approach. We performed GSE selection from a normalized library of randomly fragmented cDNA in a retroviral vector. Putative GSEs were identified after three rounds of infection, taxol treatment and insert recovery. Initial testing showed protective effect of these sequences, yet more extensive analysis produced variable results. A putative GSE derived from mitochondrial *coxIII* gene is nearly identical to an independently isolated GSE that confers aphidicolin resistance. To elucidate involvement of mitochondria in drug response, we established cell lines lacking mitochondrial DNA. These cell lines showed resistance to several anticancer drugs. To avoid the artifacts associated with selective markers used in prior selections, we developed novel retroviral vectors that use green fluorescent protein as a marker gene. A library in one of these vectors was used to initiate a new multistep taxol selection, which is currently in progress.

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Introduction.

Chemotherapeutic treatment, based on antiestrogens (such as tamoxifen) or cytotoxic chemotherapeutic drugs (doxorubicin, cyclophosphamide, methotrexate, 5-fluorouracyl) has shown limited efficacy in the treatment of breast cancer (1). The major new drug to enter the chemotherapeutic armamentarium for this malignancy in the past decade is taxol, an alkaloid extracted from the bark of *Taxus brevifolia.* In a number of clinical trials taxol-induced response rate in breast cancer varied from 20 to over 60% depending on regimen, dose and prior treatment (2). Objective response was found even in the metastatic cancer resistant to doxorubicin or other means of extensive therapy (2-4). However, a substantial number of tumors demonstrated only a partial response or no response at all. Furthermore, taxol dose escalation is limited by its side effects, primarily myelotoxicity, causing a need for an efficient strategy to protect normal tissues from possible damage. Understanding the molecular determinants of taxol sensitivity or resistance in breast carcinoma cells is likely to result in the development of chemotherapeutic regimens aimed at avoiding or reversing clinical resistance to taxol. In addition, it could lead to the development of strategies to overcome dose limiting side effects of taxol, allowing the improvement of the patients' quality of life and at the same time escalating the therapeutic dose.

The aim of the present study is to identify the genes that determine resistance or sensitivity of human cells to taxol. It is well documented that intracellular accumulation of paclitaxel causes hyperpolymerization of microtubules (5-6), prevents normal cell cycle progression and induces programmed cell death (apoptosis) (7). Known mechanisms of taxol-resistance include drug efflux by P-glycoprotein (Pgp) (5), accumulation of an anti-apoptotic protein Bcl-2 (8), and alterations in tubulin (9-11) that prevent paclitaxel binding. However, the role of Bcl-2 and tubulin alterations in clinical taxol resistance has not been sufficiently documented, and the reported incidence of Pgp positivity in breast carcinomas cannot explain the majority of resistance cases (12). In addition, the changes in tubulin functions are usually detrimental to cell growth in the absence of taxol (9,11).

Our approach to the identification of chemotherapeutic sensitivity genes is based on the isolation of genetic suppressor elements (GSEs), derived from such genes and inducing cellular resistance or sensitivity to the corresponding agents. GSEs are short cDNA fragments that counteract the genes from which they are derived by encoding inhibitory peptides or antisense RNAs (15-19). Our laboratory has previously developed the methodology for GSE selection from retroviral libraries carrying short random fragments of normalized (uniform-abundance) cDNA from mammalian cells and identified several GSEs conferring resistance to anticancer drugs or inducing neoplastic transformation. The same strategy is being used in the present project to isolate GSEs that render human cells resistant to paclitaxel. The cloned GSEs will be used to identify full-length cDNA sequences of the corresponding genes. The genes giving rise to such GSEs would constitute likely determinants of taxol sensitivity in breast

carcinoma.

Body.

1. Brief summary of the preliminary data.

A library of randomly oriented short cDNA fragments in retroviral vector pLNCX (20) has been constructed in our laboratory. For a target cell line, I have engineered HT1080 human fibrosarcoma cell line to express the cDNA for murine ecotropic receptor (21). This modification enables us to use very efficient transduction with high titer ecotropic retroviruses that are free of helper virus and not infectious to humans (22). This cell line, HT1080E14, appears to be a suitable model for drug-response in human cells, as it is sensitive to all chemotherapeutic compounds tested, including paclitaxel. A fraction of the library has been introduced into HT1080E14 cells and infected population (~$4x10^6$ independent infectants) was subjected to 2 rounds of taxol treatment. A new method for provirus rescue based on Long and Accurate PCR (23) has been developed in our laboratory (24) and used to transfer the reduced complexity provirus mixtures into fresh populations of HT1080E14 cells (secondary infectants). After another round of provirus transfer and taxol selection, PCR analysis revealed retention of 1-8 individual inserts in each experimental population. Noticeably higher survival of experimental cells as compared to vector infected control, suggested that secondary and tertiary infected populations may in fact be enriched for cells containing active GSEs.

2. Status of the project at the beginning of the year.

Putative GSEs were isolated from the cells after the final round of selection. These cDNA fragments were sequenced and compared to known human genes, as well as to the putative GSEs identified in the course of other selection projects. A putative GSE that attracted the closest attention was an anti-sense oriented fragment of human mitochondrial *coxIII* gene, which was nearly identical to the fragment isolated by Dr. E. Lausch and Dr. V. Levenson in the process of selection for resistance to replication inhibitor aphidicolin.

The recovered sequences were recloned into the original pLNCX retroviral vector and populations transduced with individual resultant constructs were generated. The cells were G418-selected to obtain pure cultures of transduced cells. Initial comparison to the vector-infected control showed higher survival of experimental cells upon taxol treatment.

As described in the attached manuscript (13), in order to avoid artifacts associated with the use of drug-resistance genes as markers of retroviral transduction we examined the utility of Green Fluorescent Protein (GFP) (28) in this role. We compared various forms of GFP, as well as different vector backbones. This allowed us to chose the constructs that provide both high level expression of a gene of interest and detectable fluorescence of transduced cells. Combining different forms of GFP allows us to simultaneously monitor multiple

transduced populations, as well as to determine the presence of several constructs within the same cells. We have also developed a fluorescence-based approach to obtain multiply transduced cells from infected populations. We demonstrated the GFP expression from our vectors is non-toxic, and suggested that dynamics of GFP-positive population may serve as an indicator of biological effects of cointroduced genes.

3. Continued testing of the putative GSEs.

We continued to investigate the effects of the sequences enriched during the previous selection experiments. We have earlier observed that some of the pure transduced populations harboring these sequences were considerably more resistant that their respective vector-transduced controls. A concern for these experiments, however, is that the examined populations were expanded and passed through G418 selection prior to testing. Potentially, prolonged separate culture of control and experimental populations, as well as the effects of G418 selection itself, could be a source of inter-populational differences. Thus, we turned to our newly developed GFP-based vector system.

In model experiments, we verified that GFP expression is neutral for cell growth and thus changes in the fraction of GFP-positive cells reflect the biological effects of cointroduced gene. When the human wild-type tumor suppressor p53 is cointroduced with GFP, green fluorescent cells are gradually eliminated from populations of p53-sensitive HT1080 cells (Figure 1), but not from populations of NIH3T3 and Rat1 cells, which are tolerant to ectopic expression of this tumor suppressor (data not shown). Noteworthy, the fraction of cells infected with an insert-free GFP vector was unchanged in either cell line.

We then cloned three sequences enriched in the course of previous taxol selection into a GFP-carrying vector LmECX. One of the putative GSEs, GSE2c1, is 100% homologous to a fragment of both translation elongation factor E1α and proto-oncogene Pti (29). The second element, GSE302, originates from an unknown gene and has a small area of homology with the human cathepsin L cDNA. The third sequence, GSE2a1, was derived from human mitochondrial gene *coxIII*, which encodes the third subunit of cytochrome *c* oxidase. We proposed that monitoring of changes in the proportion of GFP-positive cells would represent the biological effects of the putative GSEs more accurately then standard cell-killing assays performed on G418-selected populations. This analysis not only avoids prolonged cell exposure to G418, but also uninfected cells in a mixed population serve as an internal control for variations in the physiological status and conditions of cell culture.

When the newly generated HT1080 populations transduced with individual GSEs or "empty" vector were treated with taxol and monitored by FACS, in most experiments we failed to observe differences in survival between infected and uninfected cells, as exempliefied in Figure 2. In fact, the fraction of GFP-marked cells remained unchanged in the majority of treated populations, both GSE- and vector-transduced. The only assays where a transduced population appeared highly enriched after selection were several experiments conducted at a very

high dose of paclitaxel, where $<10^2$ colonies arose from $5x10^5$ treated cells (Figure 3).

Several phenomena could have accounted for the observed differences between the initial results obtained on G418 selected populations and latter results obtained on GFP-expressing cells. It is possible that the presence of the putative GSEs by itself is insufficient for protective effect, except for a fraction of cells with appropriate genetic or physiological status is achieved. Consequently, higher survival of G418-selected GSE-transduced cells could have been due to an overgrowth of individual clones with such a phenotype. Prolonged G418 selection could have contributed to this process either through allowing ample time for inter-populational differences to arise or through providing a direct advantage for otherwise rare clones. In contrast, GFP-tagged populations should be devoid of such artifacts. If the hypothesis about the need for cooperating events is correct, it can explain enrichment of GFP-tagged cells at the highest drug doses: the fraction of cells that happen to have appropriate additional genetic or physiological changes may be relatively small, and their contribution to the overall survival may be insignificant, unless background survival is minimized.

The above interpretation is in agreement with the results of a study performed by other members of our Division. As mentioned earlier, a *coxIII*-derived antisense element almost identical to GSE2a1 has been identified as a fragment enriched in the course of selection for aphidicolin resistance. Coincidental enrichment of nearly identical fragments in independent selections from a high complexity library is highly improbable. Noteworthy, the strongest protection from aphidicolin-induced killing was rendered not by *coxIII*-derived GSE alone, but by a combined expression of this fragment and three other sequences within the same cell (27; V. Levenson, E. Lausch, H. Zhu, and I.B. Roninson, unpublished).

Isolation of GSEs that act in combination is unexpected. *A priori*, under the conditions of modest transduction rate, the occurrence of cells transduced with all four cooperating inserts could be expected to be extremely low. This expectation is based on an assumption that individual viruses infect cells independently, and the probability to find multiple copies of integrated vector is determined by binomial (or Poisson) distribution. To test this prediction, HT1080-E14 cells were infected with mixtures of LNCB and LNCE viruses that are tagged with the blue and green forms of GFP, respectively (13). One of the mixtures was obtained by transfecting Bosc23 packaging cells separately with pLNCE and pLNCB, followed by combining the viral supernatants. The other was generated by simultaneous co-transfection of a Bosc23 population with these two plasmids.

Target cells were infected with either mixture at approximately the same multiplicity of infection, as verified two days post-infection by flow cytometry. Non-viable cells and particles with abnormal morphology were excluded from this analysis by combining propidium iodide exclusion criterion with appropriate gating on forward and side scatters. Flow cytometric profiles of the infected populations looked nearly identical. All four possible cells groups (negative, blue, green and double labeled) were distinguishable on double plots of blue and

green fluorescence. Notably, the fraction of double-labeled cells was in excess of what would be expected from the data on individual fluorescence detectors and this difference is statistically significant. As illustrated in Figure 4, LNCE-transduced cells have noticeably higher chance of acquiring the LNCB provirus, as compared to their LNCE-negative counterparts. This observation pointed out that, at least in our system, retroviral infection is not following binomial distribution and there may be a substantial excess of multiply infected cells over what has been previously expected.

The effect of the GSE combination conferring drug resistance was initially demonstrated in HT1080 cells exposed to a mixture of four GSE-encoding viruses. Even though our experiments suggest that multiple transduction may be a non-random event, cells harboring a combination of all four GSEs would make up only a fraction of the total transduced population. Hence the real magnitude of the effect remains obscured, unless a pure population of multiply infected cells is obtained. To obtain a more reliable insight into the spectrum and mechanism of the resistance provided by this GSE combination we took advantage of our GFP-based procedure for direct selection of multiply transduced cells. *CoxIII*-derived sequence, as well as its cooperating GSEs enriched by aphidicolin selection, were recloned into LmECX vector. A mixture of the resultant plasmids was used to generate high-titer virus. The viral supernatant was used to transduce HT1080 cells. Very high transduction rate (≅80%) ensured the presence of multiply transduced cells in either population; such cells should have the brightest GFP fluorescence. Using flow sorting, we have established a number of single-cell subclones from the brightest cells of the transduced population. We intend to determine the combination of inserts within the individual clones and correlate this data with drug resistance profiles of these cells.

We also intend to verify whether *coxIII*-derived GSEs that came from taxol and aphidicolin selection are functionally equivalent. To do so, we established transduced HT1080 populations infected either with the original aphidicolin-selected GSE combination or with the same combination in which the original *coxIII*-derived fragment is substituted for GSE2a1. Cells with various multiplicity of infection were isolated by sorting, and their resistance profiles will be compared.

As the mitochondrial *coxIII*-derived GSEs were enriched in two independent GSE selections, we took a closer look at the significance of mitochondrial functions and mitochondrial genome in drug response. Hongming Zhu in our laboratory established a number of HT1080 derivatives that are devoid of mitochondrial DNA (ρ^- variants). These cells were passed through 35 days of continuous ethidium bromide treatment in the presence of sodium pyruvate and uridine (14). Southern hybridization and PCR confirmed the absence of mitochondrial DNA in surviving clones. Two of the ρ^- clones were taken for further investigation. Drug resistance of these clones has been compared to that of the parental cell line. Increased resistance of ρ^- cells was found for several cytotoxic drugs, including, doxorubicin, one of the most commonly used chemotherapeutic agents in breast cancer (Figure 5). Further characterization of these cells is underway.

It has been suggested that p53 regulates apoptosis by modulating cellular redox potential and generation of reactive oxygen species (33). As these processes are obviously dependent on the mitochondrial functions, we are in the process of investigating whether p53 affects taxol response also in a mitochondria-dependent manner. We have generated HT1080 and HT1080ρ^- cells transduced with LXSE vector (13), or the same vector harboring anti-p53 genetic suppressor element (GSE56; 32). Dynamics of GFP-marked population of these cells, with or without drug treatment, is now being investigated.

4. New selection of taxol-resistance GSEs.

As stated in the original experimental goals of this project, we planned to repeat GSE selection starting from a more comprehensive cDNA fragment library constructed from total RNA of breast carcinoma cells. Considering our experience with GFP-based retroviral vectors, such a library was constructed in LmGCX vector by Dr. Y.Xuan in our laboratory. In the first selection experiment with the new library, the library was introduced into breast carcinoma MCF7 cells, and infected cells were subjected to low-stringency doxorubicin selection. Noteworthy, the known mechanisms of doxorubicin resistance and resistance to taxol appear to be similar. Both compounds are substrates for multidrug resistance pump P-glycoprotein. Also, both substances can cause onset of programmed cell death and the drug toxicity is alleviated by anti-apoptotic activities (8). Moreover, as both compounds are commonly used (although not concurrently) in the treatment of breast cancer, the shared cellular response mechanisms are of almost importance.

Based on the above-mentioned considerations we expected that the secondary library recovered from doxorubicin pretreated cells would be a suitable source for selecting taxol-resistance GSEs. A population of HT1080 cells was infected with this library at high multiplicity of infection and subjected to high-stringency taxol selection in parallel with vector infected cells. Survival rate of $\cong 10^{-4}$ was observed among both library- and vector-infected cells. As the starting library still retained high complexity, immediate differences in survival were not expected, although we anticipate that biologically active sequences would be considerably enriched after taxol selection. Hence, we expect differences in survival to become more apparent during subsequent rounds of selection. DNA from the surviving library-infected cells has been extracted. Currently, we are in the process of recovering the insert pool and transferring it to naive cells for further steps of selection.

Conclusions.

In the course of this work, a considerable effort has been devoted to refining and better understanding a variety of methodological and basic aspects of expression selection in retroviral vectors. These issues are critical for the experimental design and interpretation of studies aimed at identification of mediators of drug response. They also have some implications for cancer gene

therapy with retroviral vectors. In particular, we discovered that multiple transduction occurs much more frequently than could be expected from the overall transduction rates. The basis for this phenomenon is not clear, but is likely to lie in the differential susceptibility of target cells to retroviral infection or to expression of transduced sequences. This in turn may be determined by the cellular levels of the receptor protein and other cellular cofactors of infection. This may explain in part how GSEs that exhibit the strongest effect in certain combinations could be enriched during selection experiments, e.g. as the result of aphidicolin selection. Considering this possibility, we plan to modify our original scheme of GSE testing by evaluating possible GSE combinations in addition to testing the individual sequences.

The method that we developed for evaluating the biological activities of a sequence of interest by monitoring the dynamics of the transgenic fraction in a mixed population appears to be at least as sensitive as conventional resistance assays. Notably, tagging of transduced cells with GFP enables one to use the power of flow cytometry to analyzed tens or hundreds of thousands cells simultaneously, as soon as two days post-transduction. Moreover, the presence of untransduced cells on the same plate provides an excellent internal control.

We have obtained variable results during the testing of individual cDNA fragments enriched in the course of the previous taxol selection. Although random enrichment of these sequences remains a possibility, several lines of evidence argue against that. Most strikingly, a putative GSE derived from the *coxIII* gene is nearly identical to the one isolated in an independent selection for resistance to a different compound (aphidicolin). As aphidicolin resistance GSE is most active in a combination with other elements, it is possible that changes in the mitochondrial functions require some additional physiological or genetic changes for maximal effect on drug response. Presently, it is not clear whether the same or different changes are necessary for resistance to different compounds. HT1080 derivatives lacking mitochondrial DNA and individual clones harboring various combinations of potentially cooperating GSEs are now under investigation in our laboratory. Further analysis of these cells should provide more information on the contribution of nuclear and mitochondrial genes to drug response.

Overall, the appearance of mitochondria-derived sequences among putative GSEs is not surprising. Contribution of mitochondria, and cytochrome c in particular, to the onset of programmed cell death is now well documented (25-26), although the precise mechanism of this involvement is not fully elucidated. Moreover, mitochondrial transcripts were identified as RNAs selectively upregulated in taxol-treated cells (30). The mechanism of action of GSE2c1, the sequence derived from E1α translation factor or Pti protooncogene (29), in drug resistance is less clear. Possibly, it corresponds to the minimal active domain of Pti. Noteworthy, according to Cancer Genome Anatomy Project database (http://www.ncbi.nlm.nih.gov/ncicgap/) the same sequence is frequently overexpressed in cancers relative to normal tissues. Moreover, E1α has been recently shown to be a subject of regulation of p53 tumor suppressor and has a direct effect on the microtubule structure (31). Interestingly, overexpression of

E1α has an effect opposite to that of taxol: it causes depolymerization of microtubules. Identification of the parental genes for the GSEs of unknown origin will be performed once more conclusive data on the protective effect of these GSEs is obtained.

We have performed the first round of a new taxol selection. The library used for this experiment was derived from the RNA of MCF-7 breast carcinoma cells and, thus, is more likely to contain sequences with biological importance in breast cancer. In addition, the library has been constructed in a GFP-containing vector (developed in the course of this project), thus offering the unique benefits of this marker gene. In accordance with out results on non-randomness of retroviral transduction, preliminary analysis indicated that a high number, sometimes more then ten, inserts are found in individual library-transduced cells. This brings a possibility to select not only individual biologically active GSEs, but also combinations of cooperating sequences. Although effects of beneficial sequences may be attenuated by the presence of potentially detrimental inserts within the same cells, this is unlikely to be the case in this experimental population. Preselection of the library at a low dose of doxorubicin should have eliminated the chemosensitizing sequences, while still retaining considerable complexity and, perhaps, slightly enriching for biologically active GSEs. The same library is being used for other selection projects in our laboratory, and we plan to compare the sets of enriched sequences obtained in the course of all such projects. This would contribute to the task #12 of the original project, namely to compare the effects of different GSEs on resistance to various therapeutic compounds and in various cells lines.

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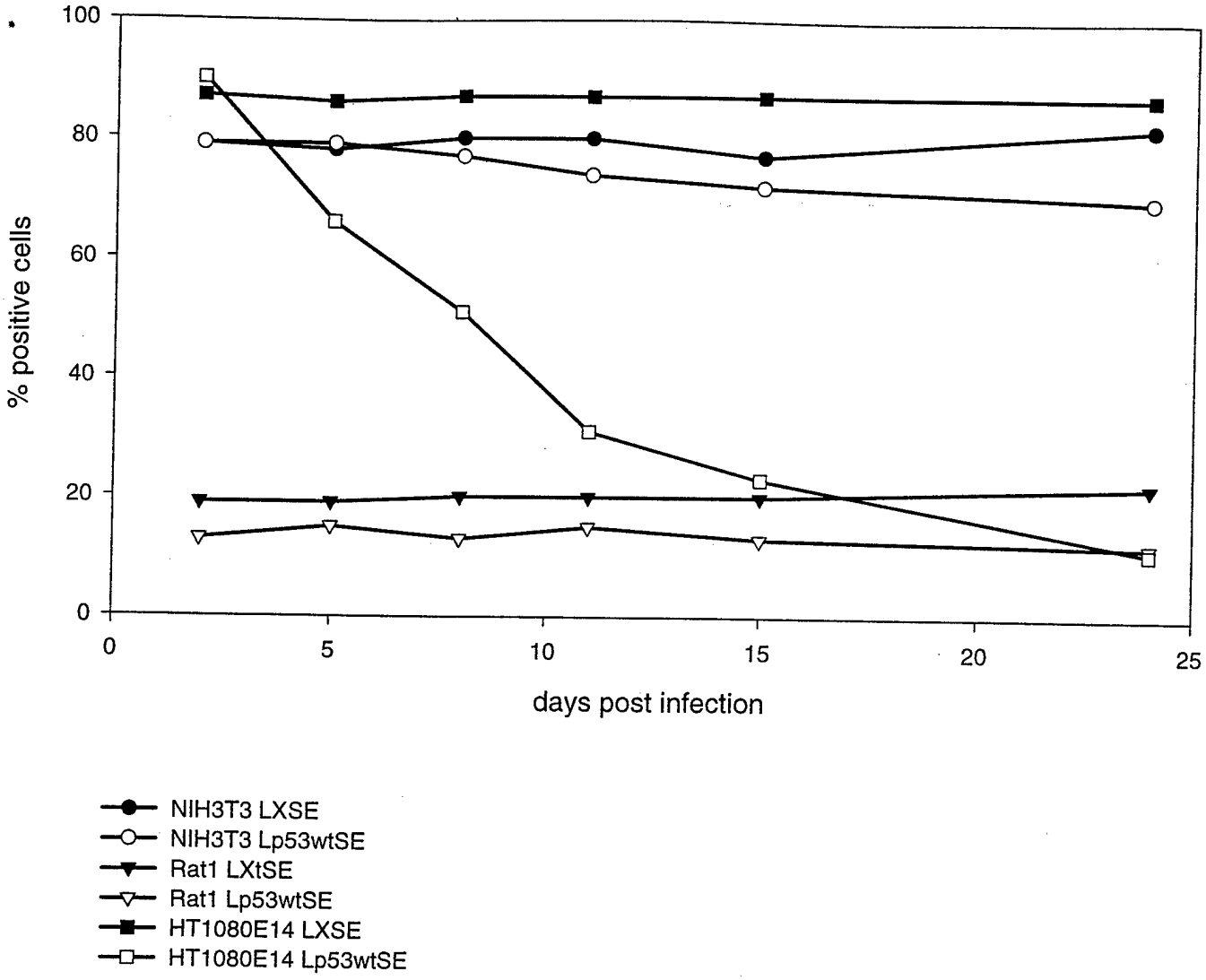

Figure 1. Differential response to p53 transduction in fibroblast cell lines verified by dynamics of GFP-positive subpopulations.

NIH3T3, Rat1 and HT1080-3'SS6 cells were infected with LXSE (13) or with the same vector carrying human wild type p53 cDNA (Lp53wtSE). Composition of infected populations was periodically analyzed in respect to GFP fluorescence via flow cytometry.

Treatment	Vector-transduced	GSE2a1-transduced	GSE2c1-transduced	GSE302-transduced
No drug	15.0%	14.4%	19.1%	16.2%
Regimen 1	14.8%	15.3%	17.8%	15.9%
Regimen 2	15.0%	15.7%	20.7%	14.7%

Figure 2. Fraction of GFP-positive cells in GSE-transduced populations upon taxol treatment.

HT1080 cells were transduced with LmECX vector (13) or with the same vector harboring GSE2a1, GSE2c1 or GSE302 inserts. $5x10^5$ cells of each population were treated with taxol. Regimen 1 included a 2-day exposure to 3ng/ml of drug. Regimen 2 included an additional replating of the surviving cells (after a 3-day recovery period) followed by a 2-day exposure to 5 ng/ml of taxol. Fraction of GFP-positive cells was measured after completion of cell dying and resumption of growth by the surviving population. Results were compared to the values in respective untreated controls. Flow cytometry was performed on Becton Dickinson FACSort instrument and the data was analyzed using CellQuest software. Only viable cells of normal morphology (as judged by propidium iodide exclusion criterion, forward and side scatter readings) were included in the analysis.

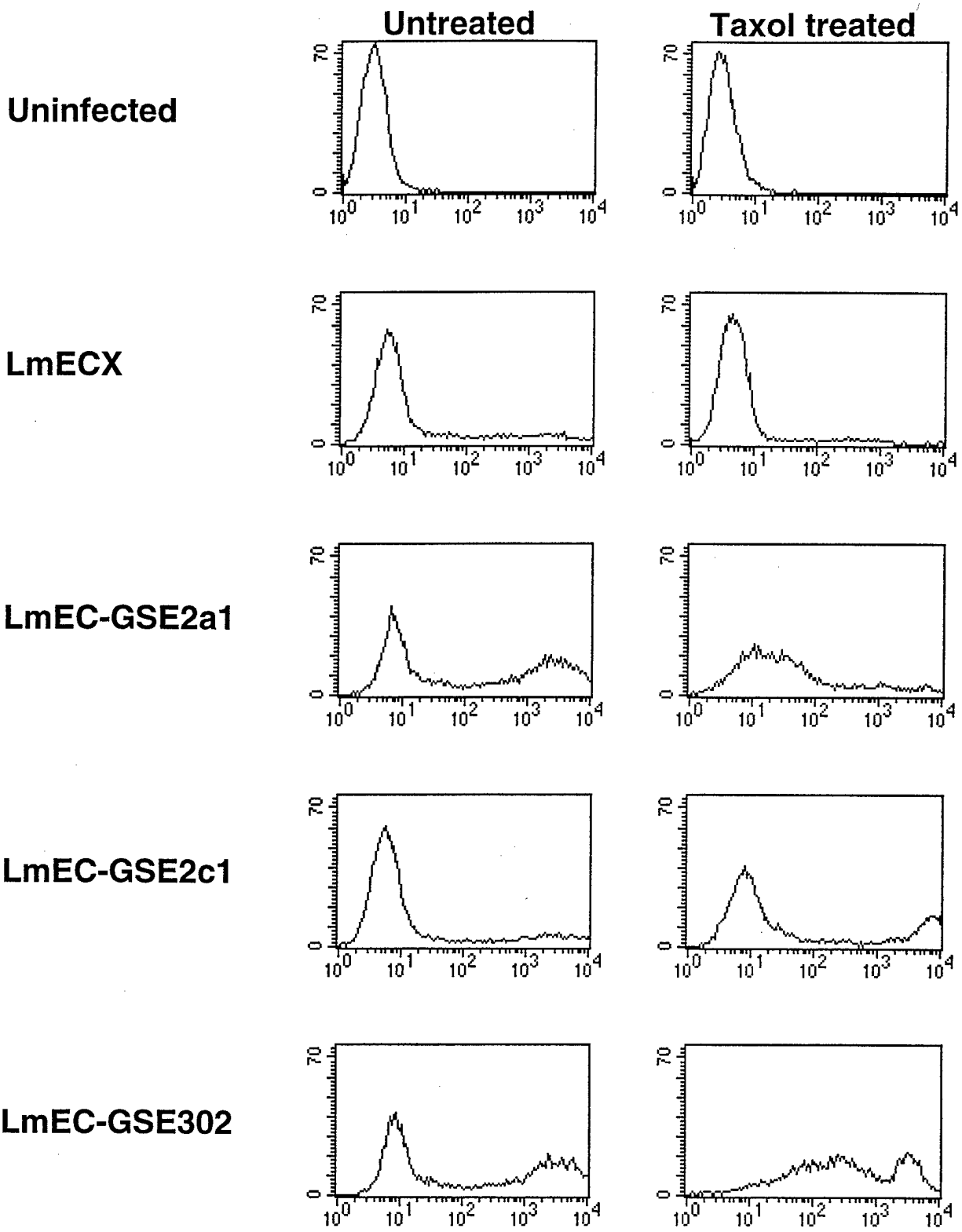

Figure 3. Effect of high-dose taxol treatment on the composition of transduced populations.

HT1080 cells transduced with LmECX vector or the same vector harboring GSE2a1 (LmEC-GSE2a1), GSE2c1 (LmGC-GSE2c1) or GSE302 (LmEC-GSE302) were treated with taxol (10 ng/ml) for 2 days and analyzed as described in Figure 2. Data is plotted as a number of cells versus fluorescence.

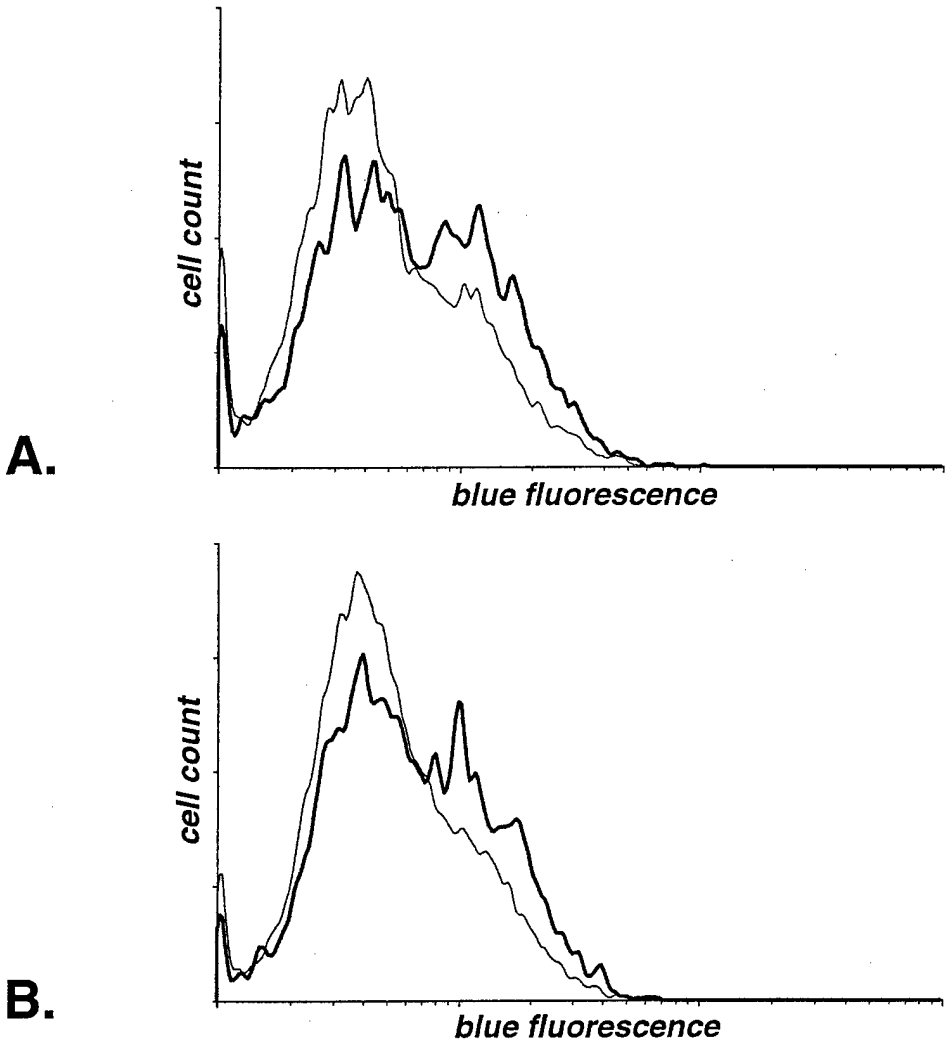

Figure 4. High efficiency of simultaneous transduction with two retroviral vectors.

HT1080-E14 cells were infected with a mixture of LNCE and LNCB viruses. In experiment A virus was produced by separate transfections of pLNCE and pLNCB into Bosc23 packaging cells followed by mixing of the viral stocks. In experiment B virus was produced by simultaneous cotransfection of pLNCE and pLNCB into the same cells. For each experiment data is shown as a comparison of blue fluorescence histograms of EGFP positive (thick line) or negative (thin line) cells. All cells were gated for normal morphology according to forward and side scatters, as well as for viability according to PI exclusion. Note the excess of LNCB transduced cells (blue fluorescent cells) among the cells transduced with LNCE.

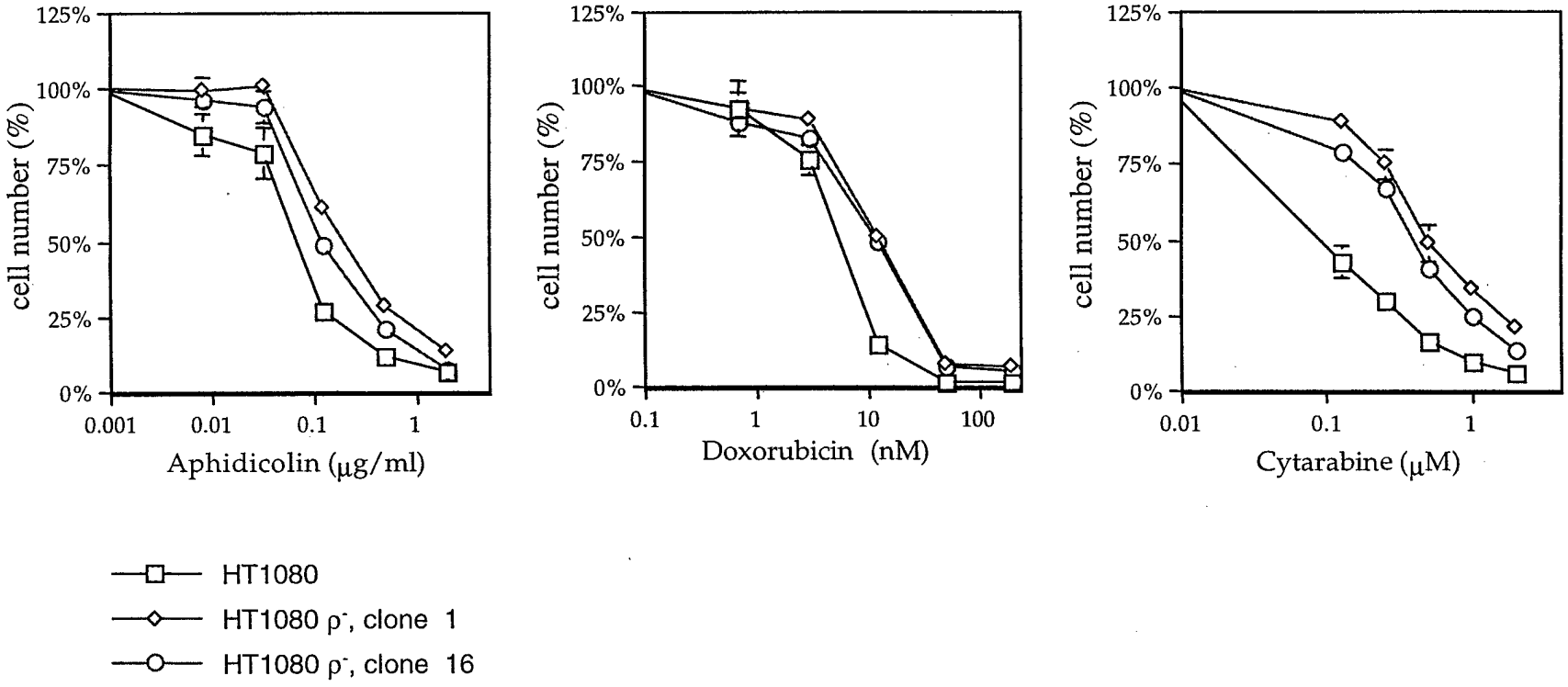

Figure 5. Drug resistance of mitochondrial DNA deficient (ρ^-) derivatives of HT1080.

Resistance of mitochondrial DNA deficient clones HT1080ρ^--1 and HT1080ρ^--16 was compared to that of parental HT1080 cell line. Cells were treated for the duration of two cell cycles with indicated doses of aphidicolin, doxorubicin and cytarabine. Three to four days post removal of the drug, cells were fixed and stained with methylene blue. The dye was extracted with 1M HCl and absorbance at 600nm (OD_{600}) was measured. The relative cell number was estimated as the ratio of OD_{600} value for each treated population to that of the respective untreated control.

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Applications of Green Fluorescent Protein as a Marker of Retroviral Vectors

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Abstract—*The Green Fluorescent Protein (GFP) of* Aequorea victoria *is used as a vital fluorescent tag for the detection and isolation of genetically modified cells. Several modified variants of GFP were tested as marker genes in retroviral vectors containing different backbones and promoter combinations. Constructs allowing for reliable detection of GFP fluorescence and the expression of a cotransduced gene from a strong promoter were identified. Cells harboring such constructs are detectable by flow cytometry, fluorescence microscopy and multi-well fluorescence reading. GFP expression in transduced cells is stable both in vitro and in vivo, and long-term dynamics of GFP-positive fractions in a mixed population can be used to monitor the biological effects of a cotransduced gene. Selection of cells with the highest GFP fluorescence enriches for multiply infected cells. The use of different GFP variants allows one to monitor simultaneously two cell populations transduced with vectors carrying GFPs that differ in their fluorescence intensity or spectral properties and to identify doubly transduced cells. In addition, transcription of an inducible promoter positioned in the opposite orientation to GFP can be monitored by the inhibition of GFP fluorescence. Thus, GFP provides a useful marker for gene transfer by retroviral vectors and extends the range of applications for retroviral transduction.*

INTRODUCTION

Retroviral transduction provides one of the most efficient and convenient means of gene delivery into animal cells. Benefits of this method include a broad spectrum of target cell types, stable integration of a transgene, predictable structure of an integrated construct and many more. Its applications extend beyond in vitro experimentation, and are being explored clinically for gene therapy. However, infection efficiency of the target cells rarely reaches 100%. Hence, for most experimental purposes it is necessary to determine the fraction of infected cells and, in many cases, obtain a pure population of infectants. For this reason, the gene of interest is usually introduced in a construct that also carries a marker gene that provides an identifiable and selectable phenotype.

The most popular class of markers consists of genes that confer resistance to various cytotoxic compounds, so that non-transduced cells can be eliminated in the course of an appropriate selection. While numerous markers of this type are available, they all share some common disadvantages. First, the efficacy of selection differs depending on the target cells and the conditions of their culture, so that the selection protocol should be optimized for each

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cell/drug combination. Second, in many cases the exposure to cytotoxic agents is undesirable, since it may affect the cell behavior in subsequent experiments. Third, it takes from several days to several weeks for a complete drug selection to occur. Fourth, the requirement for continuous expression of the drug resistance gene may favor cells that inactivated the transcription unit encoding the gene of interest, due to competition between the promoters driving this transgene or the drug resistance gene. As a result, in some cases the transduced population can be completely overgrown by clones that fail to express a transgene (1).

A recently introduced marker gene for Green Fluorescent protein (GFP) from *Aequorea victoria* (2) provides an attractive possibility to circumvent these and other limitations of conventional selectable markers. This naturally fluorescent protein (peak excitation at 376 nm, emission at 510 nm) retains fluorescence when expressed in a heterologous host. Several altered-fluorescence variants of GFP have been developed. So called "red-shifted" (488 nm excitation, 510 nm emission) variants of GFP are of particular promise, as they may be detected and isolated using the most common equipment for flow cytometry optimized to monitor fluorescein isothiocyanate (FITC) (3, 4). "Blue-shifted" (UV excitation, blue light emission) variants of GFP, "Blue Fluorescent Proteins" (BFPs), have also been developed (5–7). Due to their different spectra "red-shifted" and "blue-shifted" proteins can be detected simultaneously, although BFP requires less common detection optics.

Several groups have reported earlier successful transfer of genes for some forms of GFP through retroviral transduction (8–11). A limitation of these studies is that most of the described constructs were optimized specifically for GFP expression, while a possibility to use GFP as a marker for transduction of other genes was suggested but not explored in detail. While these groups reported no specific problems with retroviral transduction of GFP, Hanazono et al. (12) encountered difficulties in establishing high-titer producing cell lines based on PA317 packaging cells and suggested that GFP may have a cytotoxic effect and "may not be an appropriate reporter gene for gene transfer applications" (12). We have now designed, constructed and tested an extensive series of retroviral vectors that express different forms of GFP. We have compared these constructs and developed several novel applications for GFP as a marker for gene delivery, detection, isolation and characterization of cells infected with retroviral vectors.

MATERIALS AND METHODS

Plasmids and Cell Lines. LNCX and LXSN (13) were kindly provided by Dr. A.D. Miller. pBabeBleo (14) was a gift of Dr. H. Land. IPTG-regulated vectors LNXCO3, LNXCO4 and LNXRO2 were previously described (15). cDNA for the wild type GFP, GFP S65T, EGFP, EBFP was derived from pGFP1, pGFPS65T-C1, pEGFP-C1, and pEBFP-C1 plasmids, respectively (Clontech, Inc). pGreenLantern1 (Gibco BRL) was used as the source of "GreenLantern" cDNA. A mutant version of MoMuLV/MoMuSV LTR (16) was synthesized and kindly provided by Dr. W. Chen. Plasmid pCMV-P53 containing a cDNA for wild-type human tumor suppressor p53 was a gift of Dr. P. Chumakov. Details of plasmid construction are available upon request.

Bosc23 ecotropic packaging cell line (17) was a gift of Drs. W. Pear and D. Baltimore. HT1080-E14 cell line was derived by us from HT1080 human fibrosarcoma cell line after stable transfection with pBabeBleo vector (14) expressing the cDNA for murine ecotropic receptor (18). HT1080-3′SS6 is a derivative of HT1080-E14 expressing a modified LacI repressor (15). All the cells were grown in DMEM with 10% fetal calf serum at 37°C and 7% CO_2.

Transfection and Retroviral Transduction. Plasmid DNA was prepared using Qiagen Plasmid Maxi Kit (Qiagen, Inc.) according to the manufacturer instructions. Transfection of Bosc23 packaging cells was carried out as

described (17) using 10–20 μg of plasmid DNA. Target cells were plated at a density of 1–2 × 10^5 cells per P100 tissue culture plate one day before infection. The supernatant of transfected Bosc23 cells was collected 36 hrs after transfection, filtered through 0.45 nm SFCA filter (Nalgene) and added to the target cells in the presence of 4 μg/ml polybrene. When indicated, the viral stock was diluted with the growth medium. After an overnight incubation, the viral supernatant was replaced with a fresh medium.

G418 selection of infectants was carried out in the presence of 0.4 mg/ml G418 with media change every 3–4 days, until complete cell killing was observed in a non-infected control plate treated in parallel.

FACS Analysis and Cell Sorting. Cells were harvested by brief trypsinization, washed with phosphate-buffered saline (PBS) and resuspended in PBS to the final concentration of 10^5–10^6 cell per ml. FACS analysis of wild-type and red-shifted GFP variants for individual experiments has been performed using one of the three flow cytometers: FACSort, FACSVantage (both with argon laser excitation at 488nm, GFP detection using BP530/30 filter; Becton Dickinson) and Coulter Epics Elite (argon laser excitation at 488nm, GFP detection using BP525/10 filter). Propidium iodide (PI) was added to the samples to the final concentration of 1 μg/ml and PI fluorescence was used to distinguish between live and dead cells. Only live (PI-negative) cells with normal morphology (as determined by side and forward scatter) were included in the analysis. Typically 1–2 × 10^4 viable cells were analyzed. Similar results were obtained using either one of the three machines.

Simultaneous flow cytometric analysis of LNCB and LXSE infected cells was carried out with a modified Coulter Epics 753 flow cytometer. Cells were excited with blue (488nm band) and UV (predominantly 351nm and 363nm bands) light. Light from the sample first passed through a UV laser block (408LP filter). 10% reflecting mirror was used to direct light through a standard 488 band pass filter to a side scatter detector. For the passing light, 490LP dichroic mirror was used to direct shorter blue light through a 440/40BP filter for the detection of EBFP fluorescence, while the light of longer wave length was transmitted to 550LP dichroic mirror. Reflected green light was passed through 525/10BP filter and used to measure EGFP fluorescence, while transmitted red light was passed through 635/10 BP filter and used to detect propidium iodide fluorescence.

Cell sorting was carried out using FACSVantage under the conditions described above. Sorted cells were collected in DMEM with 20% fetal calf serum. Subsequently, cells were pelleted, resuspended in regular growth medium and plated on tissue culture plates. For the establishment of single cell subclones, selected cells were directly deposited onto 96-well tissue culture plates using a cell deposition unit.

Detection of GFP Expression by Fluorescence Microscopy and Multi-well Fluorescence Reading. For fluorescence microscopy cells were trypsinized, washed with PBS and deposited on microscopic slides. The microscopes used were: Nikon Labophot-2 fluorescent microscope with XC75 CCD video camera (Nikon) and DAPI (for BFP detection) or FITC (for red-shifted GFP) filter sets, and Leitz fluorescent microscope with FITC filter set (for detection of wild-type and red-shifted GFP). We have also used Olympus epifluorescent microscope with RF-2 module and FITC filter set to visualize GFP expression in cells attached to the tissue culture plates.

For GFP detection in a multi-well format, cells were seeded onto 24-well tissue culture plates (Falcon). Prior to measurement, the growth medium was substituted with PBS and the fluorescent signal was read using CytoFluor II multi-well plate reader (Perseptive Biosystems) using 485 nm excitation and 530nm emission filter sets.

Copy Number Analysis of the Integrated Proviruses. Individual cell clones were expanded and genomic DNA was extracted using Qiagen Blood and Cell Culture DNA kit (Qiagen) according to the manufacturer's recommendations. DNA was digested with KpnI.

Following digestion, DNA was purified and its concentration was measured using diphenylamine method, in triplicates for each sample. 10 μg of each sample was loaded on 0.8% agarose gel along with KpnI-digested calibration mixtures of DNA of non-infected cells and pLXSE plasmid, prepared at the ratios corresponding to 1 and 2 copies of pLXSE per diploid amount of DNA. Upon electrophoresis, samples were transferred onto HybondN (Amersham) nylon membrane. The NheI/BglII fragment of pEGFP-C1 containing the cDNA of EGFP was used as a probe after labeling with ^{32}P by random priming using the Multiprime DNA Labeling System (Amersham). Hybridization and high-stringency washing were carried out as previously described (19). To determine the provirus copy number in test samples, radioactive signal from individual bands was quantified using Betascope 603 Blot Counter (Betagen Corp.) and divided by the signal generated by the pLXSE plasmid in the calibration mixtures.

RESULTS AND DISCUSSION

Comparison of GFP-Expressing Retroviral Vectors. The retroviral backbones in our constructs have been derived from the earlier published vectors (13–15). We originally started by cloning the wild-type GFP cDNA from pGFP1 plasmid (Clontech, Inc) into LNCX (13) retroviral vector. The resulting pLNCGfp plasmid was used to transfect Bosc23 packaging cells (17) and the viral supernatant was used to infect murine NIH3T3 fibroblasts and human HT1080-E14 fibrosarcoma cells. Transfected and infected cells were analyzed using fluorescent microscopy or flow cytometry under conditions optimized for the detection of FITC. In neither case were we able to detect fluorescent signal (data not shown), suggesting that the wild-type GFP was not efficiently excited by the blue light in our available equipment.

Various "red-shifted" GFP variants gradually became available over the course of this study. GFP S65T (from pS65T-C1; Clontech) contains an amino acid substitution that brings excitation peak of this protein close to 488 nm, a property ideally suited for detection using conventional argon lasers as well as other equipment developed for the detection of FITC. In addition, GFP S65T was reported to have improved folding rate of the chromophore, as compared to the wild type GFP. "Green Lantern" GFP (from pGreenLantern1; GIBCO BRL) contains the same S65T mutation, as well as optimization of certain codons in order to avoid those that are particularly unfavorable for translation in human cells (20). "Enhanced GFP" or "EGFP" (21) (from pEGFP-C1, Clontech) was engineered to include S65T change as well as complete optimization ("humanization") of the codons and additional mutations (22) that improve folding and reduce aggregation. In addition to the red-shifted variants, we have also used BFP (from pEBFP-C1, Clontech) which contains the Y66H mutation that changes the fluorescence spectrum (5–7) together with mutations improving folding and chromophore formation (22), as well as complete "humanization" of the coding region.

Figure 1 shows the structures of different GFP-containing retroviral vectors that we generated, as well as their relative fluorescence (RF) values, determined after retroviral transduction into derivatives of HT1080 cells. Since the most important characteristic of a fluorescent marker is the separation achieved between positive and negative cells, the RF is defined as a ratio between median fluorescence of GFP-positive and GFP-negative cells. The absolute fluorescence, as measured by FACS, may change depending upon the setup of the instrument (e.g. laser power or amplification parameters for the detectors). However, the RF of FACS-distinguishable distinct cell populations remains essentially the same regardless of the changes in the absolute values. This allows us to compare results obtained using the same FACS with the same cell line, but at different times. The use of median, rather than mean, fluorescence values makes RF relatively insensitive to the appearance of rare particles with abnormal fluorescence as well as to minor experimental varia-

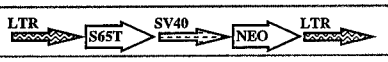
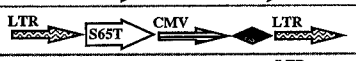
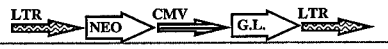
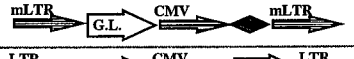
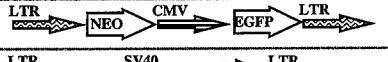
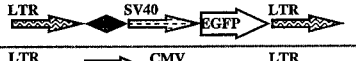
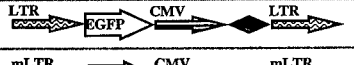
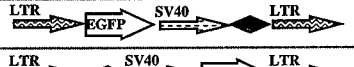
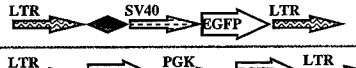
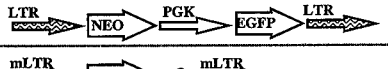
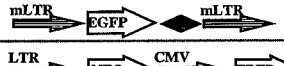
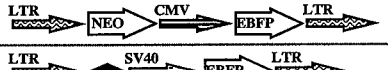
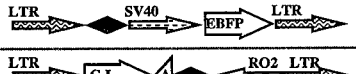
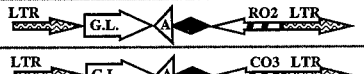
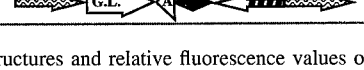
Vector structure	Name	Relative Fluorescence
	LNCR	10-20
	LRSN	10-30
	LRCX	2-3
	LXCR	10-20
	LNCG	100-200
	LXSG	15-20
	LGCX	7
	LmGCX	8-10
	LNCE	200-600
	LXSE	20-40
	LECX	10
	LmECX	12-15
	LESX	50-65
	BabeE	20
	LNPE	10-20
	LmEX	150
	LNCB	N.A.
	LXSB	N.A.
	LGXRO2	20
	LGXCO3	8
	LGXCO4	8

Fig. 1. Structures and relative fluorescence values of GFP-containing retroviral vectors. Vectors are shown as integrated proviruses. Relative fluorescence (RF) was determined in HT1080 cells transduced with the corresponding viruses, using excitation with an argon laser on Becton Dickinson FACSort flow cytometer (EBFP is not fluorescent under these conditions). RF for LGXRO2, LGXCO3 and LGXCO4 was determined in the absence of β-galactosides. A range of RF is shown for the most extensively tested vectors. LTR—Moloney Murine Leukemia/Sarcoma Virus Long Terminal Repeat; mLTR—modified LTR with an extra Sp1 binding site (16); CMV—human cytomegalovirus promoter/enhancer region; SV40—Simian Virus 40 promoter/enhancer region; PGK—murine phosphoglycerate kinase gene promoter; RO2—modified Rous Sarcoma Virus Long terminal Repeat with 2 *lac*-operators (15); CO3,CO4—modified CMV promoters with 3 and 4 *lac*-operators respectively (15); neo—neomycin phosphotransferase gene; S65T—GFP S65T from pS65TC1 (Clontech); G.L.—"Green Lantern" GFP from pGreenLantern (Gibco BRL); EGFP—"Enhanced GFP" from pEGFPC1 (Clontech); EBFP—"Enhanced Blue Fluorescent Protein" from pEBFP (Clontech); X—proposed cloning sites (vary from vector to vector); A—polyadenylation signal from Herpes Simplex Virus thymidine kinase gene.

tions in gating. For a given construct, the RF may be affected by several factors. One of them is autofluorescence of uninfected cells, which in its turn is affected by culture conditions and the diluent used to prepare cells for analysis (e.g. growth medium or PBS). Only the results obtained in PBS were included in Fig. 1. The second factor that influences RF is the infection rate. Populations infected at higher rates exhibit higher median fluorescence, probably as a result of multicopy integration (as shown below). For this reason, results obtained at infection rates higher than 50% were generally excluded from the table. Another parameter affecting RF is the length of time in culture from infection to analysis. We have observed that maximal RF is achieved between 48 and 72 h after infection, although fluorescent cells can be detected much earlier. Hence, Fig. 1 contains data only from analysis done 48 or more hours post infection. For the most extensively analyzed constructs, a typical range of RF is given. RF values presented in this paper were obtained using argon laser excitation (488nm). BFP is not fluorescent under these conditions, but, as described below, it was detectable by flow cytometry with UV excitation or fluorescent microscopy. Comparison of the RF values for red-shifted GFP variants in similar retroviral backbones yielded the following hierarchy: EGFP > "Green Lantern" > GFP S65T (compare LNCR to LNCG and LNCE; Fig. 1). While this article was in preparation, similar ranking of GFP variants (EGFP > "humanized" GFPS65T, "red-shifted" GFP > wtGFP) was reported by others (23).

The other major determinant of RF is the promoter which drives GFP expression. We consistently observed high levels of GFP expression from the human CMV promoter, as determined both by FACS and fluorescent microscopy. In fact, we faced an unexpected problem when doing FACS analysis on cells infected with the LNCE vector (Fig. 1): due to extremely high fluorescence level, positive cells were hard to analyze on the same scale with the uninfected cells. This high fluorescence level allows us to identify unambiguously and isolate cells that are infected or transfected with such vectors, even when such cells comprise only a fraction of a percent in the total population.

Somewhat lower level of expression was observed from the vectors in which GFP expression was driven by the SV40 promoter (compare LNCG to LXSG and LNCE to LXSE; Fig. 1). Noteworthy, we observed a small but detectable decrease in fluorescence provided by LXSE carrying an insertion in its cloning sites, but this decrease did not jeopardize FACS-based detection of transduced cells (data not shown). This observation suggests, however, that when a drug resistance gene rather than GFP is used as a selectable marker, the expression of this gene and hence the toxicity of the selective agent may be different for cells transduced with insert-containing and insert-free vectors. Thus, a vector containing a biologically inert insert may be a more accurate negative control than an insert-free vector, unless GFP is used as a selectable marker.

The murine phosphoglycerate kinase (PGK) promoter provided only a low fluorescence in the LNPE construct (Fig. 1). However, the PGK promoter is known to be stably expressed in a very broad variety of murine tissues (24), and therefore in some cell types it might be more useful than in HT1080 cells.

We were especially interested in developing GFP vectors analogous to a commonly used neo-containing vector LNCX (13), where the potent CMV promoter would drive the expression of the non-selected gene of interest, while GFP would be expressed from the LTR promoter. We have encountered considerable difficulties, however, in using LRCX vector, the first vector of this type, where the RF of the S65T GFP turned out to be substantially lower than in any other construct tested (Fig. 1); LRCX-transduced cells were essentially indistinguishable from their non-infected counterparts. We have tried three different cloning strategies to obtain a functional vector of LRCX structure,

and numerous individual plasmid clones were analyzed (data not shown), yet none of the constructs yielded sufficient fluorescence for our applications. We cannot attribute this to an insufficient activity of the LTR promoter per se, since LRSN vector containing the same LTR-GFP cassette but carrying SV40 rather than CMV as the internal promoter provides relatively strong fluorescent signal (Fig. 1). Similarly, the LESX vector carrying EGFP has provided much brighter signal than LECX (Fig. 1). Our results suggest that the CMV promoter interferes with LTR-driven expression of GFP (compare LmECX to LmEX; Fig. 1). This is in agreement with our previous observations that G418 selection of cell populations infected with an LNCX-based vector leads to the accumulation of cells with inactivation of the CMV-driven gene (1). Despite the failure of LRCX, its analogs carrying improved versions of GFP, LGCX and LECX, provided acceptable levels of RF. These levels were further improved by the use of a mutant version of MoMuLV/MoMuSV LTR, which contains a new Sp1 site in its U3 area (16), in the LmGCX and LmECX vectors (Fig. 1). These vectors allow one to take advantage of the strong CMV promoter for the expression of the gene of interest.

Aside from the above-described vectors based on the LN retroviral backbone (13), we have also inserted GFP into pBabe vector backbone (14). Vectors of pBabe series carry a variety of resistance markers as well as some modifications for improved virus packaging. We have modified pBabeBleo to incorporate EGFP in place of *bleo*. The resulting construct, pBabeE, is similar to the LN-based pLXSE vector, yielding similar titer of infectious virus (not shown) as well as fluorescence of infected cells (Fig. 1). The pBabeE plasmid is smaller than pLXSE and is based on a higher copy replicon that simplifies manipulations with this vector in plasmid form. In addition, the availability of both pLXSE and pBabeE extends cloning options by providing two vectors with similar structure but different sets of restriction sites.

The observations summarized in Fig. 1 suggest some possibly general predictions for gene expression from retroviral vectors expressing one gene from the LTR and the second from an internal promoter in the same orientation. While these predictions are based primarily on the results obtained in human fibrosarcoma cells, they are in agreement with our observations in a number of other cell types, including murine and rat fibroblasts and human breast carcinoma cells (unpublished observations). First, vectors containing an internal SV40 promoter are likely to express both genes at similar levels (compare LXSE to LESX). Thus, such vectors as LXSG, BabeE, LXSE, LESX and LXSB are well balanced in terms of relative expression of both transcriptional units. In contrast, vectors carrying an internal CMV promoter would probably favor very high expression of the downstream gene but relatively low of the upstream gene (compare LRCX to LXCR and LECX to LNCE). We suggest that LGCX, LmGCX, LECX and LmECX would be the vectors of choice when the highest expression level of the CMV-driven gene of interest is desired, while the ease of detection of GFP-expressing cells is not a priority. In contrast, LNCG, LNCE and LNCB may be used (after substituting *neo* for the fragment of interest) when it is desired to have the highest possible fluorescence, although transgene expression from LTR may be lower than from the CMV promoter.

Long-Term Stability of GFP Expression. To investigate whether GFP expression from our vectors would have any detrimental effect on cell viability or proliferation, we infected a population of HT1080-E14 cells with LNCG virus and continuously propagated the infected population in the absence of any selection. The composition of the population in respect to GFP fluorescence was periodically determined by FACS. After more than three months of culture (>115 cell doublings) the fraction of GFP-positive cells sustained only minor changes (Fig. 2A) while the relative fluorescence of the positive cells remained essentially the same

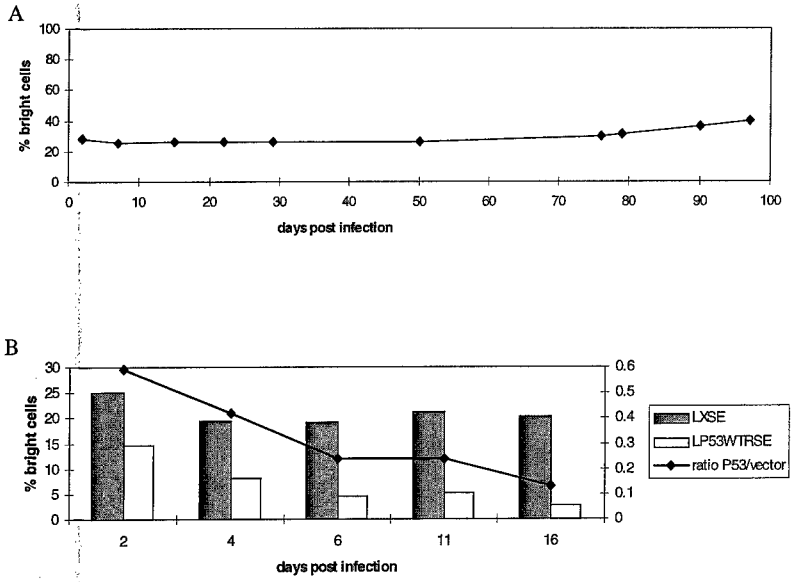


Fig. 2. GFP stability in transduced cells. Panel A. Long-term stability of GFP-positive fraction in a mixed population. HT1080-E14 cells infected with LNCG virus from Bosc23 packaging cells were continuously propagated in the absence of selection. The percent of GFP-positive cells was periodically determined by FACS analysis. Panel B. Demonstration of the growth-inhibitory effect of p53 tumor suppressor through changes in the GFP-positive fraction. HT1080-3′SS6 cells were infected in parallel with either LXSE or LP53WTRSE (wild type p53 cDNA in LXSE vector) virus, in duplicates. The percent of GFP-positive cells was periodically assayed by FACS analysis. Standard errors for the duplicates were <10% of the mean in all cases. The decreasing ratio of the fractions of GFP-positive cells between LP53WTRSE and LXSE demonstrates a gradual loss of infected cells in which GFP was cotransduced with the tumor suppressor.

(data not shown). Similar observations were made with several other cell types infected with GFP vectors (20–30 days monitoring), including murine NIH 3T3 and rat Rat1 fibroblasts, and MCF7 human breast carcinoma cells (E.S.K., B.S., Y. Xuan, and I.B.R., unpublished).

To test the stability of coexpression of GFP and a cotransduced gene, we infected HT1080-E14 cells with LNCR virus, yielding a 9% GFP-positive population. Subsequently, one part of the infected population was selected for *neo* gene function by growth in the presence of G418. FACS analysis of the resistant cells was carried out upon completion of selection (10 days post infection), and revealed >93% GFP-positive cells. The other part of the population was used after infection to isolate GFP-positive cells by FACS sorting, after which GFP-positive cells were assayed for colony formation in the presence and in the absence of G418. Sorted cells remained almost 100% positive for GFP upon reanalysis two weeks after sorting and their plating efficiency with and without G418 was essentially the same, indicating close to 100% positivity for *neo* expression.

To verify that GFP does not interfere with cell growth in vivo and is detectable within a tumor, we injected a mixture of HT1080-E14 cells, both non-infected and infected with LNCG, subcutaneously into a nude mouse. Three weeks later, the growing tumor was excised and pieces of it were viewed under a fluorescent microscope. As expected, the specimen represented a mixture of fluorescent and non-fluorescent cells (Fig. 3, A and B). A part of a tumor was trypsinized and plated in tissue culture. After one passage in culture, recovered cells were subjected to FACS analysis revealing presence of GFP-positive cells with characteristically bright fluorescence (Fig. 3C). Thus, GFP-expressing cells can be readily identified

Fig. 3. GFP expression does not interfere with tumorigenicity and is detectable in tumors grown in vivo. HT1080 cells were infected with LNCG virus and expanded without selection. 2×10^6 cells were subcutaneously injected into a nude mouse. Three weeks later the tumor was removed and analyzed under a Leitz fluorescent microscope in a bright field (A) or with FITC filter set to detect GFP fluorescence (B). A part of the tumor was trypsinized and explanted in cell culture. After one passage in culture the cells were analyzed by FACS, revealing the retention of a bright fluorescent population (C). Results of the flow cytometric analysis are shown on the contour plot of fluorescence versus forward scatter with 2% threshold and one smoothing iteration.

within tumors and recovered for subsequent manipulations.

The above results indicate that GFP expression is stable both in vitro and in vivo and has no detrimental effect on cell growth. Our observation of the stability of GFP expression in vivo is in agreement with the reports that GFP transgenic animals are viable and lack signs of major abnormalities (25). Furthermore, stable production of a GFP-containing retroviral vector has been successfully achieved in PA317 cells (9), suggesting that the reported failure of GFP expression in this cell line (12) is unlikely to be due to the GFP toxicity. Although we cannot exclude that extremely high expression of GFP, as of almost any protein, may be detrimental to a cell, we believe that inability to obtain stable GFP-expressing cells is more likely to result from inadequate expression, selection or detection systems.

Use of GFP to Monitor Growth Effects of a Cotransduced Gene. Given the stability of GFP expression, we hypothesized that changes in the GFP positive population may serve as a sensitive indicator of the effects of a cotransduced gene on cell growth. To test the applicability of GFP to monitor cellular effects of a tumor suppressor gene, we infected HT1080–3′SS6 cells with LP53WTRSE (wild type p53 cDNA in LXSE vector) or with insert-free LXSE vector and monitored FACS profiles of the infected populations (in duplicates) over a period of 16 days. We have observed a gradual decrease in GFP-positive cells when GFP was cointroduced with p53 (from ~15% at day 2 to ~2% on day 16), while the fraction of GFP positive cells infected with the LXSE control remained essentially unchanged (Fig. 2B). This phenomenon was reproduced in a number of similar experiments that differed in the initial transduction rate (10–95%) and duration of observation (16–28 days) (data not shown). Thus, periodic monitoring of the GFP-positive fraction in the infected cell population should provide a convenient and sensitive means to detect the effects of different genes on such phenotypes as growth rate and cellular response to various treatments.

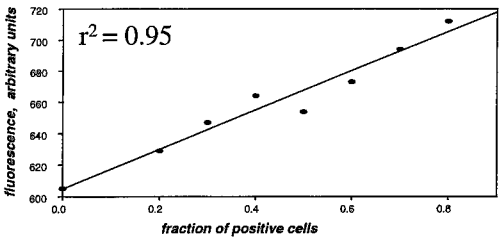


Fig. 4. Detection of transduced cells using a multi-well fluorescence plate reader. Different numbers of HT1080-3′SS6 cells transduced with LNCG virus and selected with G418 were added to non-infected cells to produce mixtures of various composition. For each mixture, two wells of a 24-well cell culture plate were seeded with 50,000 cells. The next day the growth medium was substituted for PBS and the fluorescent signal was read on CytoFluor II multi-well fluorescence plate reader (Perseptive Biosystems) using 485 nm excitation and 530 nm emission filter sets. The graph demonstrates the correlation between the fraction of LNCG-transduced cells and the fluorescence of each cell mixture (shown as an average of duplicate wells).

Quantitation of GFP Fluorescence with a Multiwell Fluorescence Reader. While most of our work relied on FACS for the detection of cells transduced with GFP retroviruses, we tested if the fluorescence of such cells would be high enough to be detected in living cells by a multiwell fluorescence reader. Different numbers of HT1080-3′SS6 cells transduced with LNCG virus and selected on G418 were added to non-infected cells to produce mixtures of various compositions. The graph in Fig. 4 demonstrates the correlation between the fraction of LNCG-transduced cells and fluorescence

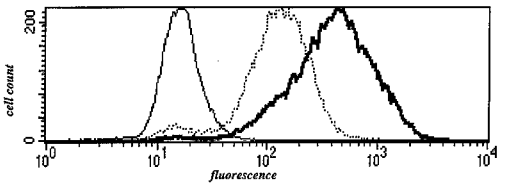
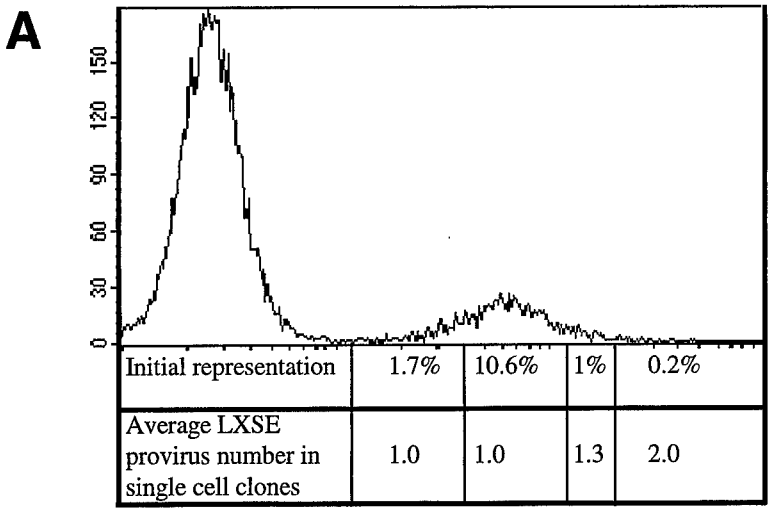


Fig. 5. Fluorescence profiles of HT1080-E14 populations infected at different concentrations of LRSN virus. HT1080-E14 cells were infected with LRSN virus from Bosc23 packaging cells with (dotted line) and without (solid line) 100-fold dilution of the viral stock. Following G418 selection, cells were analyzed by FACS using FACSort (Becton Dickinson) and compared with non-infected HT1080-E14 cells (fine line).



Initial representation	1.7%	10.6%	1%	0.2%
Average LXSE provirus number in single cell clones	1.0	1.0	1.3	2.0

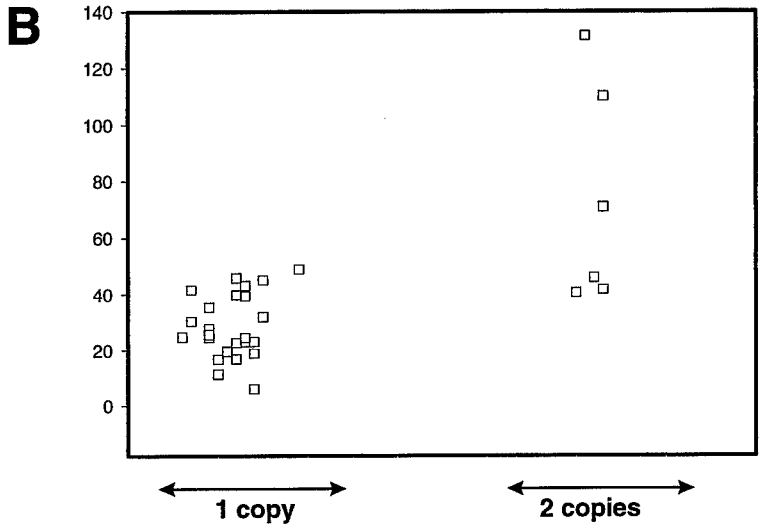
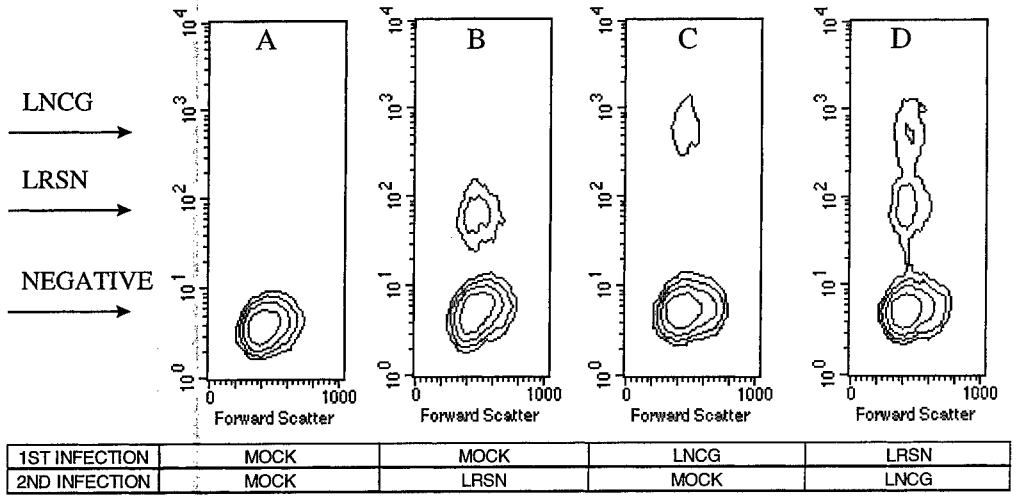


Fig. 6. Brighter LXSE infectants contain a higher number of integrated proviruses. Panel A. Fluorescence-based selection for higher-copy integrants. HT1080-3′SS6 cells were infected with LXSE virus from Bosc23 packaging cells. The histogram shows the fluorescence profile of the infected population. The four indicated positive areas were separated by flow sorting, and single-cell subclones were established from the sorted fractions. The copy number of integrated proviruses was estimated in individual subclones by Southern hybridization with an EGFP-specific probe. Panel B. Correlation between relative fluorescence and copy number of integrated proviruses in individual clones. Median fluorescence of individual clones, containing one or two copies of the integrated provirus was measured by flow cytometry and normalized for the medium fluorescence of uninfected cells.

of each cell mixture. The observed correlation is significant ($r^2 = 0.95$) and was reproduced in a number of similar experiments. The ability to detect and estimate the fraction of GFP-positive cells in a population using a multiwell fluorescence reader suggests that this simple procedure may be used to facilitate large-scale titration of recombinant retroviruses.

Fluorescence-Based Selection for Higher-Copy Integrants. We have previously observed



1ST INFECTION	MOCK	MOCK	LNCG	LRSN
2ND INFECTION	MOCK	LRSN	MOCK	LNCG

Fig. 7. Simultaneous detection of cells transduced with two different "red-shifted" GFP variants. HT1080-3′SS6 cells were mock-infected (panel A) or infected separately with LRSN or LNCG vectors (panels B and C) or consecutively with both vectors (panel D), and analyzed by FACS two days after the final infection. The results are presented as contour plots of fluorescence (Y axis) versus forward scatter (X axis) with one smoothing iteration and 3.2% threshold.

that cells transduced with retroviral vectors at different infection rates express the transduced genes at different levels, due to multicopy provirus integration at higher infection rates (1). To verify this observation using GFP vectors, HT1080-E14 cells were exposed to native and 100-fold diluted LRSN viral supernatant, yielding approximately 80% and 5% infection efficiencies, respectively. Upon completion of G418 selection, these two populations were compared by FACS (Fig. 5). The population initially transduced at a higher rate retained significantly higher median level of fluorescence, in agreement with the previous observations (1). This result underscores that a comparison of biological effects between different recombinant viruses should be performed at similar infection rates. It also indicates that comparison of GFP fluorescence in different constructs should be conducted at low infection rates, when the infectants are more likely to contain only a single copy of each provirus.

To isolate cells co-infected with different retroviruses or when higher expression of a retrovirally transduced gene is desired, it is advantageous to isolate a population of cells harboring more than one integrated provirus. We hypothesized that such a population may be obtained after infection with GFP vectors, through FACS selection of the brightest cells. We also expected that, unlike an increase in the stringency of drug selection (1), selection of brighter cells would not provide an opportunity for rare rearranged clones to overgrow the rest of the cells. To test these predictions we infected HT1080-3′SS6 cells with LXSE virus, with an infection rate of 13.5%. We then carried out FACS analysis on the infected population,

Fig. 8. Simultaneous detection of cells transduced with "red-shifted" and "blue-shifted" GFP variants. The results of FACS analysis carried out using Coulter Epics 753 flow cytometer are shown as density plots; X axis—green fluorescence, Y axis—blue fluorescence. Panel A. uninfected HT1080-3′SS6 cells. Panel B. HT1080-3′SS6 cells transduced with LNCB retrovirus and selected with G418. (Note a small but detectable fraction of EBFP-negative cells). Panel C. HT1080-3′SS6 cells, clone LXSE2-27 (a single-cell subclone of LXSE-transduced cells). Panel D. Cells of the clone LXSE2-27, superinfected with LNCB virus and selected with G418. Panel E. mixture of cells from B and C. Panel F. mixture of cells from A, B, C and D.

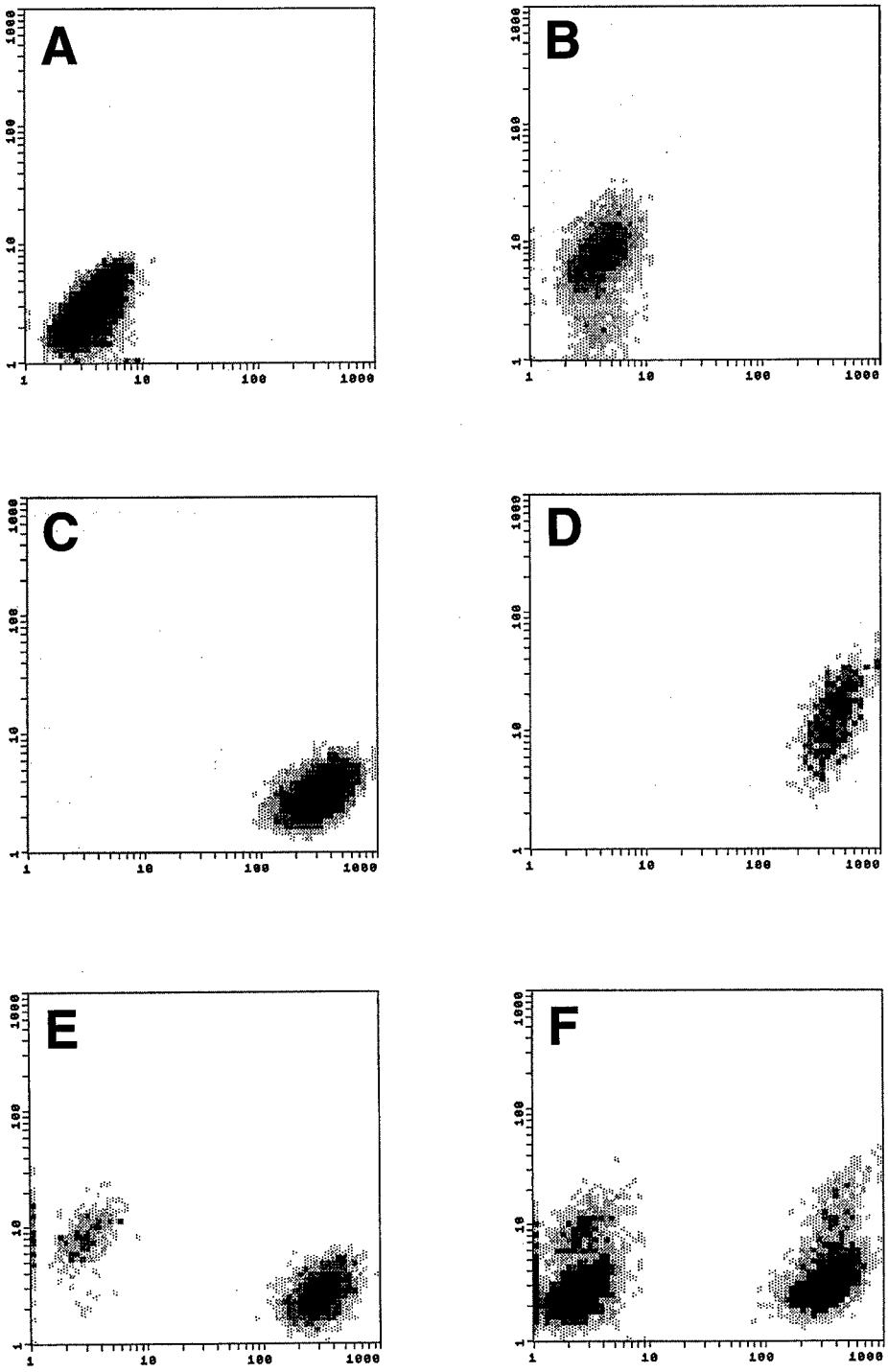


Fig. 8.

sorted out four different areas of the positive peak (Fig. 6A) and established a number of single-cell subclones from each of these areas. The copy number of integrated proviruses in each clone was estimated by Southern blotting and hybridization with an EGFP-specific probe. The average value obtained for each group of clones (3–12 clones in each group) is shown in Figure 6A. In agreement with our predictions, the brightest cells carried two proviruses, in contrast to the predominantly single-copy integrants in the rest of the positive peak (Fig. 6A and B), and no rearranged proviruses were detected in any of the clones by Southern hybridization (data not shown). Clones carrying either one or two copies of the provirus showed considerable variation in their relative fluorescence (Fig. 6B). This result emphasizes that comparison of gene expression by different vectors is more accurate if done on mass populations rather than individual clones of transduced cells.

Simultaneous Monitoring of Two Vectors Containing Different Forms of GFP. Many types of molecular genetic studies could benefit from the ability to monitor simultaneously two cell populations transduced with different genes. We have demonstrated the feasibility of using different forms of GFP as distinguishable fluorescent tags by simultaneous detection of two red-shifted variants of GFP characterized by different levels of cellular fluorescence. We have infected HT1080 cells with LRSN virus and then superinfected one half of these cells with LNCG. The superinfected cell population was compared by FACS to cells infected with LRSN alone, LNCG alone and the non-infected control. Figure 7 demonstrates that LRSN- and LNCG-infected cells form two distinct fluorescent populations. Although LNCG-infected and doubly-infected cell populations could not be distinguished from each other in the flow profiles, quantitative analysis of this experiment (not shown) revealed that LRSN-infected cells are efficiently superinfected with LNCG, since the fraction of the LRSN-positive population is reduced upon infection with LNCG.

An alternative approach to visualizing two different GFP-labeled cell groups at the same time is to use GFP forms with spectral properties that are sufficiently different to allow for independent observation. For this purpose, we constructed LNCB and LXSB vectors that contain the gene for BFP under the control of CMV promoter. Cells infected or transfected with these constructs were visualized as blue under fluorescent microscope equipped with a DAPI filter (data not shown). Also, cells expressing "blue", "red-shifted" or both GFP variants were distinguished from each other and from the non-infected cells by flow cytometry with simultaneous UV and blue excitation (Fig. 8). Besides obvious applications for simultaneous monitoring of cells infected with two different vectors, the availability of both EGFP and EBFP in similar vectors extends the opportunity for combination studies with other fluorophores. For example, EBFP (but not EGFP) can be used in combination with FITC, while EGFP (but not EBFP) can be used in combination with Hoechst dyes. In addition, both proteins can be combined with red dyes (such as propidium iodide) or orange dyes (such as phycoerythrine).

Use of GFP to Monitor the Expression of Promoters Transcribed in the Opposite Orientation. We have recently described inducible retroviral vectors that carry β-galactoside-regulated promoters, positioned in the opposite orientation to the LTR (15). When using such vectors, it is important to be able to monitor not only their presence in the cells but also the proper regulation of the inducible promoters. We hypothesized that in these constructs induction of an internal promoter would downregulate LTR-driven expression of a marker gene, due to antisense inhibition. Consequently, such changes in the expression of the marker might serve as an indicator for appropriate regulation of an inducible promoter.

To verify this prediction we have substituted *neo* with the "Green Lantern" GFP in three retroviral vectors carrying different β-galactoside-inducible promoters (15). The

resulting constructs (LGXRO2, LGXCO3 and LGXCO4; Fig. 1) were used to infect HT1080-3′SS6 cells that express LacI repressor modified for nuclear localization in mammalian cells. In the absence of inducers, the RF values of these vectors (Fig. 1) were inversely correlated to the previously characterized basal expression levels of the corresponding inducible promoters (15). To test if the induction of these promoters would decrease the GFP fluorescence, we infected HT1080-3′SS6 cells with LGXCO3 and selected GFP-positive cells. Upon short expansion, one half of the collected population was propagated in the presence and the other half in the absence of IPTG. Flow-cytometric comparison revealed a noticeable difference between induced and non-induced cells, with the induced cells showing greatly decreased fluorescence (Fig. 9). Subsequent return of the induced cells into IPTG-free medium brought their fluorescence to the level similar to that of the non-induced cells. As expected, changes in the fluorescence were inversely correlated with the changes in the expression from the internal modified CMV promoter, as measured by a RT-PCR assay (data not shown). Although several approaches exist to use GFP expression to monitor a regulated promoter (26), only in our system GFP fluorescence is detectable even when the gene of interest is not activated. Therefore, GFP can serve both as a marker and as a reporter for delivery and expression of potentially cytotoxic or cytostatic genetic elements.

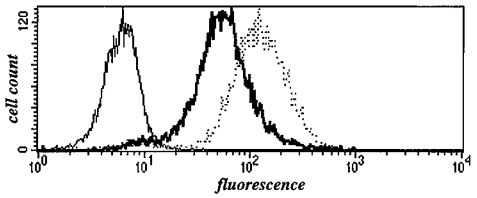


Fig. 9. Change of GFP expression in response to the activation of internal promoter in LGXCO3 vector. HT1080-3′SS6 cells were infected with LGXCO3 virus, GFP-positive subpopulation was collected and split into two parts. One part was cultivated in the presence, and the other in the absence of the inducer (5 mM IPTG). The histogram shows the fluorescence profiles of induced (solid line) and non-induced (dotted line) GFP-positive cells compared to the non-infected control (thin line). Induction of the modified CMV promoter was independently confirmed by RT-PCR (not shown).

In conclusion, we have designed and characterized an extensive series of retroviral vectors that express different variants of GFP. These vectors provide a broad choice of promoter combinations, expression levels and detection options to be applicable to a diverse range of experimental protocols. These vectors are useful for gene delivery, tagging and tracking of specific cell groups within complex populations, optimization of transfection and transduction protocols, as well as for the studies of basic aspects of retroviral transduction. The use of GFP as a marker gene in retroviral constructs yields insights into structure-functional organization of retroviral vectors and extends the range of experimental goals achievable through retroviral transduction.

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